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Isolation and Characterization of the Nuclear Matrix From the Male Xenopus laevis Following Estrogen Administration: Kinetics of [^{3}H] Uridine Incorporation

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At various times following estorgen administration, the nuclear matrix was isolated from the liver of male Xenopus laevis by sucrose gradient centrifugation of nuclei treated with a high-salt buffer and DNase I in the presence of a proteolytic inhibitor (PMSC – phenylmethyl sulfonyl chloride). Electron micrographs of the nuclear matrix demonstrate a sponge-like network attached to a well-defined inner envelope with a ribosome-free outer envelope. Chemical analyses show that the HSB-DNase-treated nuclei consist of 16% DNA, 2% RNA, and 82% protein, a composition that is consistent with that of nuclear matrices isolated from other species. The specific activity of the matrix-associated RNA following estrogen treatment appears to be maximally enhanced after 5 h and decreases until approximately 12 h, when the activity begins to increase again.

Key words: nuclear matrix, estrogen, RNA, uridine, Xenopus laevis

Several investigations have demonstrated evidence of the existence of a ribonucleoprotein (RNP)-matrix structure in the nucleus of a variety of cell types [1–3]. Faiferman and Pogo [1] have postulated that the nuclear matrix is involved in the processing and/or transport of messenger RNA (mRNA) in eukaryotic cells. To study the relationship between the nuclear matrix and heterogeneous nuclear RNA (HnRNA) in the processing and transport of mRNA, a system is desired in which the synthesis of a specific mRNA could be initiated in significant magnitude. Such a system is found in the estrogen-induced synthesis of vitellogenin in the male Xenopus laevis [4–6].

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In this study, we have described the isolation and characterization of the nuclear matrix from the liver of the male Xenopus laevis. Evidence is presented that a RNP-matrix structure is present in the nucleus of the male X laevis and that hormone administration results in rapid enhancement of incorporation of [^{3}H]uridine into RNA that is associated with the nuclear matrix structure.

METHODS

Treatment of Animals

Male X laevis (50 g) were injected in the dorsal lymph sac with 125 μCi of [5-^{3}H]-uridine (sp. act., 25 Ci/mmoles; Schwarz/Mann, Orangeburg, New York) 40 min prior to sacrifice after various times of estradiol-17β administration (1 mg/0.1 ml propylene glycol). Nuclei were isolated by a modification of the method of Risley et al [7]: 7 g of liver was homogenized in 35 ml of buffer A (3 mM $CaCl_2$, 2 mM Tris-HCl (pH 8.0), 5 mM sodium bisulfite, and 1 mM phenylmethyl sulfonyl chloride (PMSC)) in a 100-ml Glenco glass-Teflon homogenizer (6 strokes) at 95 V. The homogenate was centrifuged at 900g for 10 min, and the pellet resuspended in buffer A and centrifuged as before. The nuclear pellet was then resuspended in 65% metrizamide (Accurate Chemical and Scientific Corp., Hicksvill, New York) using a Dounce homogenizer to a final concentration of 58% (N_D^{20} = 1.4262) and centrifuged at 7,700g for 20 min. The nuclear pellicle was resuspended in 2.3 M sucrose in buffer A, layered over 7 ml of 2.4 M sucrose in buffer A, centrifuged at 25,000 rpm for 60 min in a Beckman SW27 rotor, washed in buffer A, and finally centrifuged at 900g for 10 min.

Isolation of Nuclear Matrix

Isolation of the nuclear matrix was essentially as described by Miller et al [8]. Isolated nuclei were washed three times in TMS buffer (25 mM Tris-HCl, pH 7.5 (23°C), 2.5 mM $MgCl_2$, 0.25 M sucrose) containing 3 mM PMSC. The nuclei were centrifuged at 900g and the final nuclear pellet was suspended in a 2.5 vol. of a low-salt buffer (LSB) (0.2 M NaCl, 5 mM MgCl, 25 mM Tris-HCl, pH 7.6 (23°C), and 5 mM PMSC). To aliquots of 1 ml was added 1 ml of a high-salt buffer (0.9 M NaCl, 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.6 (23°C), and 5 mM PMSC), giving a final ratio of packed nuclei to extraction buffer of 1:5; the final concentration of the buffer (HSB) was 0.5 M NaCl. The nuclear suspension was gently stirred with a glass rod and then incubated for 2–3 min with 300 μg of DNase at 37°C. This suspension was briefly centrifuged for 7 min at 900g, and the supernatant was applied to a 12-ml, 30–60% sucrose gradient in HSB buffer, then centrifuged in a Spinco SW41 rotor at 18,000 rpm for 18 h. Gradients were fractionated into 0.25-ml fractions and the absorption of each measured at 260 nm. To each fraction, 5 ml of 10% cold trichloroacetic acid (TCA) was added, and next the acid-insoluble material was collected on glass-fiber filters (Whatman GF/A, Whatman, Inc., Clifton, New Jersey), washed 2 times with cold 10% TCA, dried, and added to 5 ml of TT-21 scintillation cocktail (Yorktown Research, New Jersey). Radioactivity was measured in a Beckman LS-230 scintillation counter.

Electron Microscopy

Pellets of HSB/DNase/PMSC-treated nuclei were resuspended in 2 ml of fetal calf serum and centrifuged at 500g for 30 min, 4°C. Pellets were then fixed for 4 h in Karnovsky's fixative and postfixed for 1 h in 1% osmium tetraoxide. The pellets were dehydrated

in alcohol, embedded in Epon 812, and sectioned on a Sorvall MT-2 microtome. The grids were stained initially in 2% uranyl acetate for 30 min and then in lead citrate for 5 min. Micrographs were taken on a Siemens IA.

Chemical Analysis

RNA was determined by the method of Fleck and Munro [9], DNA by the method of Burton [10], and protein by the Bio-Rad protein assay (Bio-Rad Technical Bulletin 1051, 1977).

RESULTS

To assess the integrity of the liver nuclei of X laevis, each progressive step in the HSB-DNase-PMSC treatment for the isolation of the nuclear matrix was monitored by light and electron microscopy (EM). As shown in Figures 1 and 2, the electron micrographs, following the matrizamide isolation and TMS wash, demonstrate nuclei of approximately 4 μ in diameter, an intact double membrane with ribosomes attached to the outer membrane, and a prominent nucleolus. However, there appears to be some bleaching of the chromatin during the TMS wash, as seen in Figure 2. As a result of the HSB treatment (Fig. 3) most of the chromatin appears to be removed as well as the ribosomes of the outer membrane. The nuclear matrix of X laevis is now revealed as the characteristic meshwork structure extending throughout the nucleus and attached to the nucleolus, inner membrane, and

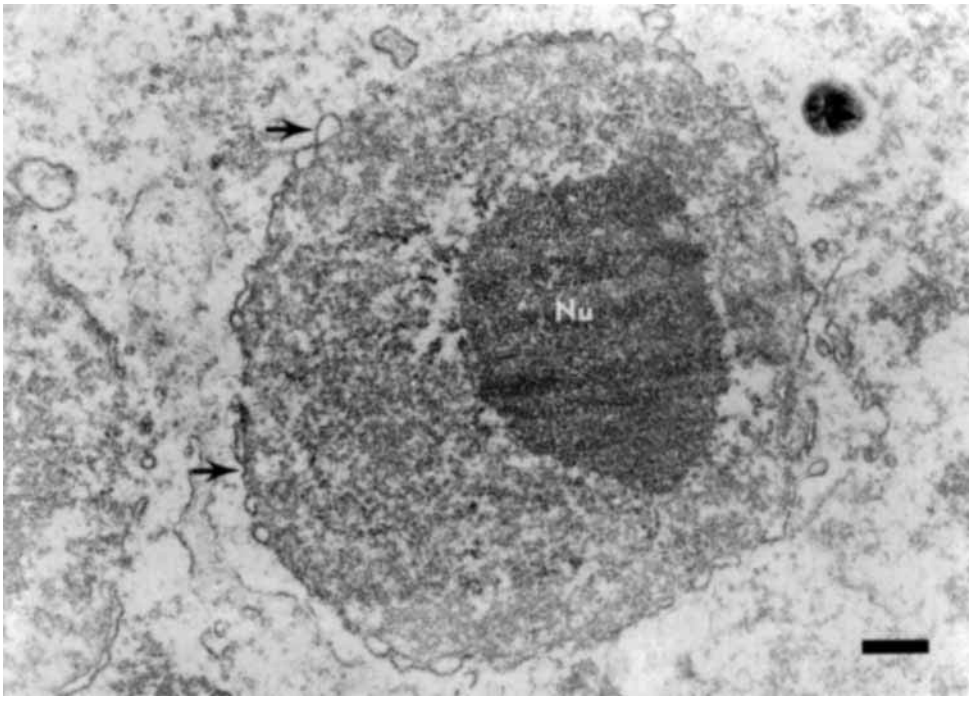

Fig. 1. X laevis liver nucleus after metrizamide isolation. A prominent nucleolus (Nu) and double nuclear membranes with ribosomes (arrows) attached to the outer membranes are observed. × 24,000; bar 0.5 μ.

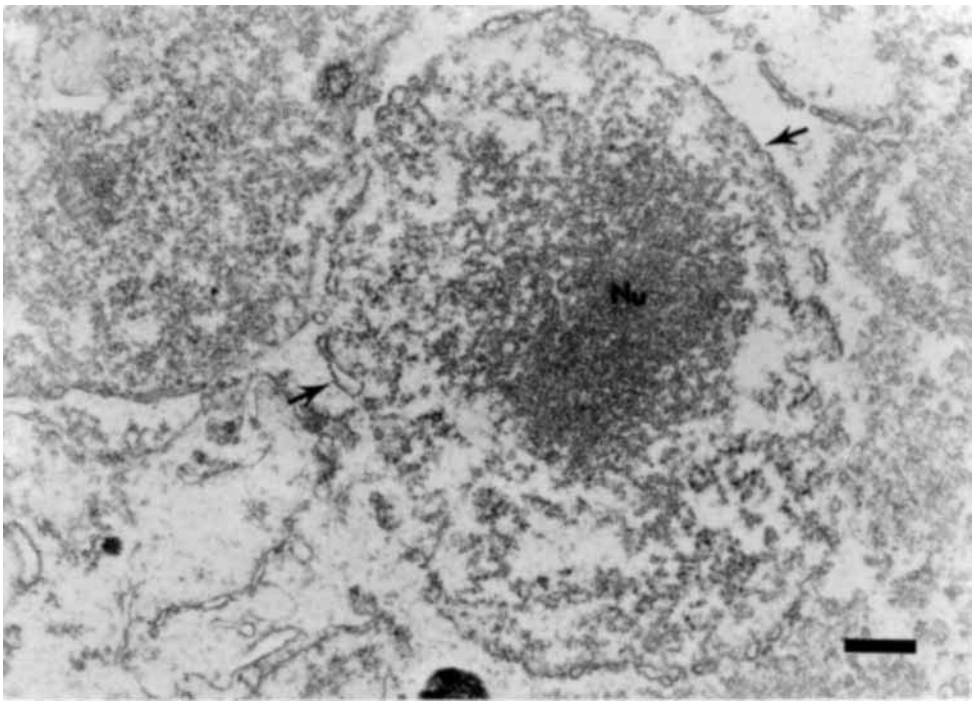

Fig. 2. X laevis liver nucleus after TMS buffer wash. The nucleolus (Nu) and ribosome-studded outer membrane (arrows) are still intact; however, some bleaching of chromatin has occurred. × 26,000; bar 0.5 μ.

nuclear pore complexes. Preparation of the matrix structure results in a 70% reduction in the size of most nuclei. This reduction in nuclear size has also been observed by others [3, 11, 12]. In Figure 4, the final nuclear product of the extraction procedure results in a more electron-dense nuclear matrix. The outer membrane of this structure seems to bleb out, sometimes becoming detached between the dense nuclear pore complexes. The dense unattached aggregates seen along the periphery of the outer membrane are believed to represent fragments of nuclear matrices from nuclei ruptured during the process of isolation and fixing. Table I shows that the nuclear matrix structure seen in the electron micrographs is consistent in composition of protein, DNA, and RNA with the nuclear matrix demonstrated in various other species: approximately 75% protein, 15% DNA, and 5% RNA [3, 8]. Hormone treatment did not have any apparent effect on the morphology of the nuclear matrix structure.

When the HSB/DNase/PMSC-treated nuclei of X laevis are fractionated on a 30–68% sucrose gradient after 18 h at 18,000 rpm in a Spinco SW41 rotor, a prominent band of UV absorbing material (seen as a turbid, sharply defined band) occurs at a density of 1.31 g/ml (64% sucrose). These values compare favorably to 1.25 g/ml (54% sucrose) reported for rat liver nuclear matrix (achromatinic) structures [8]. When observed by EM, this band consists of nuclear matrix bound within a double, blistering membrane appearing fragmented at times, and is identical to the nuclear matrix shown in Figure 4. The majority of [^{3}H]uridine incorporation following a 40-min pulse was found associated with nuclear matrix structures on sucrose gradients and the amount of labeled material associated with

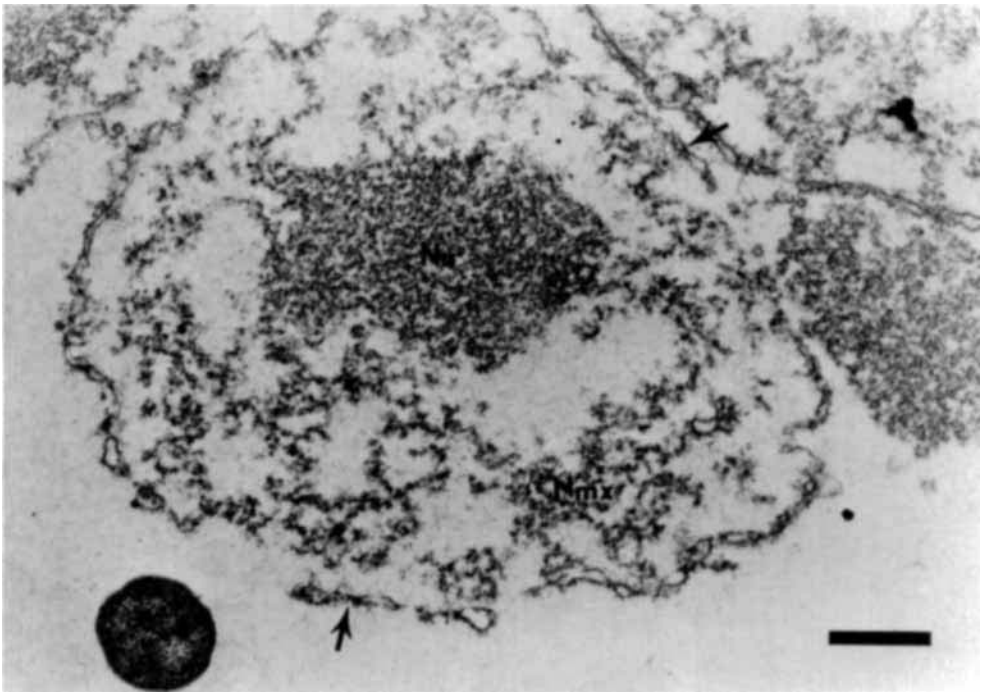

Fig. 3. X laevis liver nucleus after HSB treatment. The nucleolus (Nu) is still intact; however, the outer membrane ribosomes have disappeared, leaving the nuclear pores (arrows) intact. Most of the chromatin is absent, leaving behind a meshwork structure extending throughout the nucleus and attaching to the nucleolus and inner membrane. This is the first ultrastructural evidence of the X laevis nuclear matrix (Nmx) showing a clear attachment to the nuclear pores. × 38,000; bar 0.5 μ.

the nuclear matrix is shown to vary as a function of time following estrogen stimulation, as demonstrated in Figure 5. The radioactivity associated with the nuclear matrix was found to be highly sensitive to ribonuclease digestion. Digestion of the nuclear matrix with ribonuclease T1, T2, and A resulted in a 96% loss of trichloroacetic acid-precipitable radioactivity. In order to clearly demonstrate the kinetics of RNA labeling during the first 12 h of stimulation, data are presented in Figure 6 in the form of specific activity of the nuclear matrix isolated at a density of 1.31 g/ml. Enhancement of uridine incorporation is detected after only 1 hr of estrogen administration, and maximal incorporation occurs by approximately 5 h. The levels of uridine incorporation then decrease until approximately 12 h, at which time it appears that the rate again begins to increase. From these data, one can see a >50-fold enhancement of incorporation of [^{3}H]uridine into the nuclear matrix of the male X laevis after 5 hr of estrogen administration.

DISCUSSION

This study demonstrates the isolation of a nuclear matrix from the liver of the male X laevis. The nuclear matrix extends throughout the nucleus, attaching to the nucleolus, inner membrane, and nuclear pore structures; the composition was determined to be 82%

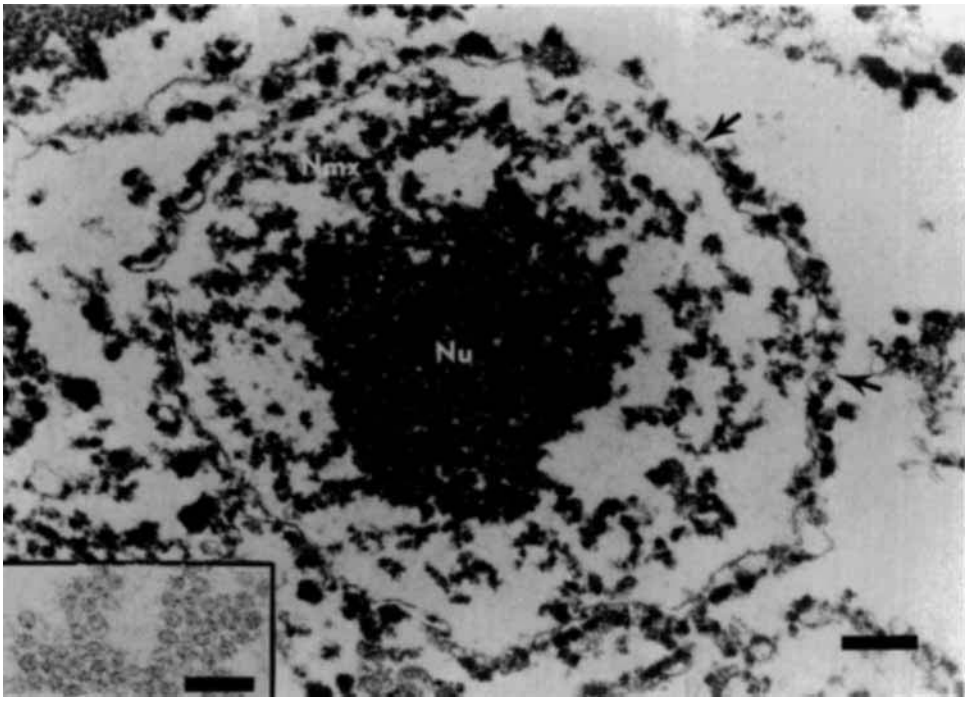

Fig. 4. X laevis liver nucleus after HSB/DNase/PMSC treatment. The same structures as noted in Figure 3, except the dense clumps seen outside the nuclear membrane, are believed to be fragments of nuclear matrix from ruptured nuclei. As shown in the insert, circular structures having an approximate outer diameter of 300 Å can be observed. These structures resemble nuclear pore complexes en face. The outer circle appears to be interconnected with similar surrounding structures and encloses a small circular structure. × 28,000; insert × 133,000; bar 0.5 μ and 0.1 μ, respectively.

TABLE I. Protein, DNA, and RNA Biochemical Analysis

	Protein			DNA			RNA		
	mg/gm[a]	%[b]	% recovery[c]	mg/gm	%	% recovery	mg/gm	%	% recovery
Untreated nuclei[d]	12.00	53	–	10.36	46	–	0.24	1	–
Treated nuclei[e]	2.00	82	17	0.40	16	4	0.05	2	20

[a]Units are mg per gm of liver.
[b]Protein + DNA + RNA = 100%.
[c]Treated nuclei as a percentage of untreated.
[d]Nuclei after metrizamide isolation.
[e]Nuclei after metrizamide isolation, HSB/DNase/PMSC extraction, and migration to 1.31 g/ml (64% sucrose) in a 30–68% sucrose gradient after 18 h at 18,000 rpm in a SW41 rotor.

protein, 16% DNA, and 2% RNA. As shown in Figure 4, the HSB/DNase/PMSC-extracted nuclear matrix reveals circular structures with an outer diameter of approximately 300 Å and is reminiscent of nuclear pore complexes seen en face [13, 14]. The structure and composition of the X laevis nuclear matrix isolated by this method is in agreement with other investigations in rat liver cells [1, 8, 11, 15, 16] and also in HeLa cells [3, 12].

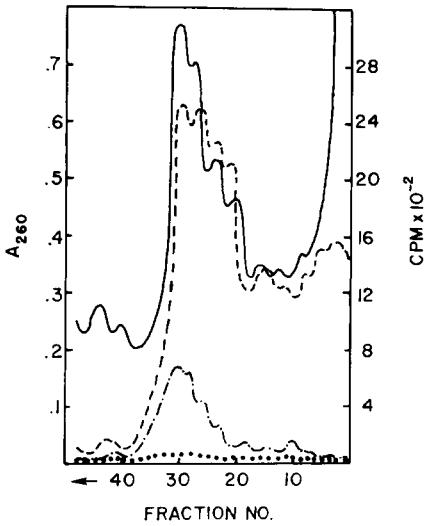

Fig. 5. Sucrose gradient analysis of HSB/DNase/PMSC-extracted male X laevis hepatocytic nuclei. A prominent, sharply defined band of absorbance (——) migrating to a density of 1.31 g/ml (64% sucrose) in a 30–68% sucrose gradient after 18 h at 18,000 rpm in a SW41 rotor was demonstrated in the liver nuclear matrix fraction at various times of estrogen stimulation for a group of 3 frogs (1 mg per frog). The cpm of five separate groups of frogs without estrogen administration was found to be 65 ± 30 (· · · · ·). The cpm at 1 h (– · – · –) and 5 h (– – –) were determined for one group of frogs each. Arrow indicates direction of sedimentation.

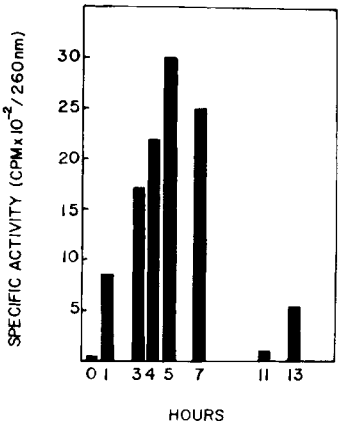

Fig. 6. [^{3}H]uridine incorporation into the male X laevis nuclear matrix after various times of estrogen administration in vivo. Each value represents a single determination from a group of three frogs except for the zero time point (no estrogen administration) which represents five separate determinations, yielding a value of 58 ± 20. [^{3}H]uridine was administered for 40 min prior to sacrifice (see Methods).

The nuclear matrix of the male X laevis hepatocyte is associated with rapidly labeled RNA in a manner similar to that demonstrated in rat liver nuclei [1, 8, 16]. During a 40-min pulse labeling, it has been demonstrated in X laevis that only minor amounts of [^{3}H]-uridine are incorporated into ribosomal RNA (rRNA), no incorporation into transfer RNA (tRNA), and no change in precursor pools [17, 18]. Furthermore, Tata and Baker [17] have shown that a subnuclear fraction, rich in nucleoli, acquires only small amounts of [^{3}H]uridine during this time and that the administration of estrogen to male X laevis does

not cause any major quantitative shift in the rate of RNA labeling in various subfractions of the nucleus.

In rat liver hepatocytes, rapidly labeled RNA associated with the nuclear matrix is composed of HnRNA and only minor amounts of rRNA [1, 8]. This rapidly labeled RNA is localized at the extranucleolar sites on the nuclear matrix, as indicated by autoradiography studies of rat hepatocytes [19] and HeLa cells [20].

When one compares the kinetics of the induced appearance of cytoplasmic mRNA for vitellogenin (cvmRNA) in estrogen-treated male X laevis [4, 5] to that of the incorporation of RNA into the nuclear matrix as indicated in Figure 6, the two appear to be closely related. The incorporation of [^{3}H]uridine into cvmRNA, as demonstrated by Baker and Shapiro [4] and Ryffel and Weber [5], is most rapid between 3 and 9 h after estrogen stimulation and is followed by a decrease until around 12 h, when another enhancement in the rate of incorporation begins. A similar temporal pattern is seen in the incorporation of [^{3}H]uridine into the nuclear matrix as demonstrated by this study. However, it must be emphasized that additional experiments are necessary to establish that the rapidly labeled RNA does represent vitellogenin message.

It is evident from the micrographs that the nuclear matrix extends outward from the inner membrane and nuclear pores. Lenk et al [21] have isolated a skeletal-like structure in the cytoplasm (cytoskeleton) which extends throughout the original outline of the cell. They also suggest that most of the polysomes are associated with this cytoskeleton by connection to mRNA. Small nuclear RNA (SnRNA) has been shown to be associated with the nuclear matrix [16, 22]. These structures are found not only in the nucleus but also in the cytoplasm associated with the endoplasmic reticulum (ER). Goldstein and Ko [23] have demonstrated that certain species of SnRNA can shuttle from the nucleus to the cytoplasm. Therefore, these observations suggest that a structural continuity, allowing for the processing and transport of RNA, may exist between the nuclear matrix and a similar structure in the cytoplasm found along the endoplasmic reticulum.

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